Exercise 4. Quorum sensing and quorum sensing inhibition

Introduction

Many Gram negative bacteria, including *Pseudomonas aeruginosa*, *Pseudomonas aureofaciens, Serratia liquefaciens*, *Agrobacterium tumefaciens*, and *Erwinia carotorova* are able to regulate gene expression in accordance with population density in a process termed quorum sensing (QS). These organisms utilize N-acyl homoserine lactones (AHLs) as signal molecules (Fig. 1). During growth the AHLs accumulate, and when a certain threshold concentration is reached, target genes of the QS systems are activated (Fig. 1). The core of a QS system consists of two genes; an I gene encoding the AHL synthase and an R gene encoding a protein able to bind AHL and DNA. The N-terminal part of the R protein binds AHL and the C-terminal parts bind DNA. Most, but not all known R proteins act as transcriptional activators upon AHL binding. Some R proteins are repressors which are derepressed upon AHL binding. Most R proteins can be activated by a range of AHLs, but the cognate AHL usually has the highest affinity. The AHL signal molecule consists of two parts, a homoserine lactone ring and a fatty acyl side chain. The AHL molecules differ in the length of the side chain, which can be either unsaturated or saturated, and contains either a hydroxyl or an oxo group at the third carbon. Most AHLs are believed to diffuse across the bacterial cell wall, with the exception of long-chain AHLs, such as the oxo-C12 AHL produced by *P. aeruginosa*, that utilize an efflux pump for translocation across the cell membrane.

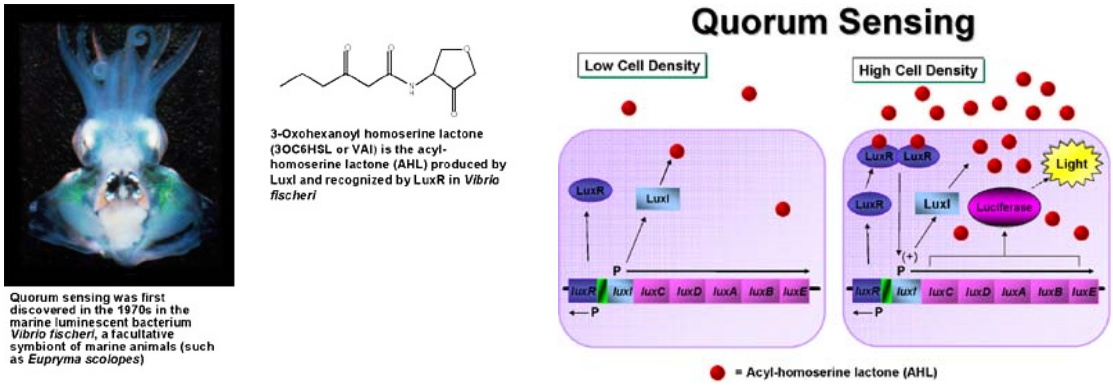


Figure 1. Quorum sensing signal molecule and activation mechanism from *Vibrio fischeri*. AHL concentration increase when the cell density is high. This results in a shift of the LuxR equilibrium towards its AHL-bound, active state. AHL binding leads to dimerization of LuxR and binding to the lux box, a 20-base pair inverted repeat located in the Plux promoter.

QS-controlled genes often encode virulence factors and gene products required for bacteria–host interactions. In addition, there is growing evidence that QS influences more complex behavioural processes such as the ability to form surface-associated structured consortia referred to as biofilms. Biofilm formation plays an important role in bacterial pathogenesis and is a common cause of persistent infections. Bacteria in biofilms are resistant to disinfectants, antibiotics and the action of host immune defences. It should therefore be possible to attenuate bacterial pathogenesis by interfering with bacterial QS systems. Knowledge about the molecular mechanisms involved in QS is important since it may form

the basis for the development of new therapeutic strategies against infections.

In the present exercise we will learn how to investigate bacterial QS systems through modern molecular biology techniques. We will use bioassay systems to monitor extracellular QS AHL molecules as well as screen for QS inhibitors which can block QS process. In the molecular biology part, we will use the non-pathogenic *Pseudomonas aureofaciens* as our model strain since the exercise-lab is not classified to do molecular genetics in opportunistic pathogens. The techniques used are of course the same as those used in investigations of real pathogens. In the QS inhibitor screening part, we will use *P. aeruginosa* as our model strain.

Here is some background of *P. aureofaciens*.

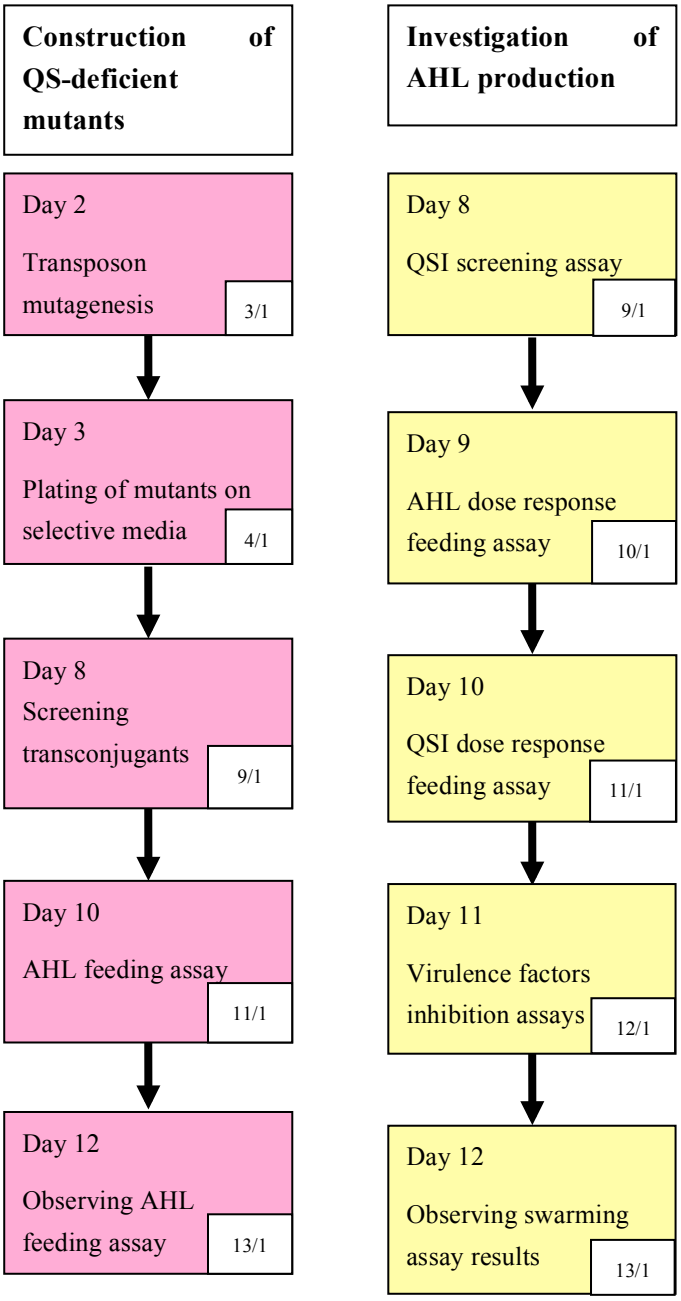
Figure 2. Phenazine in *P. aureofaciens* colony on plate

P. aureofaciens produce proteases, sideophores, chitinases, HCN, phenazines and other extracellular products that are believed to account for its ecological competitive properties. In *P. aureofaciens* 30-84, the synthesis of these antimicrobial metabolites is controlled by a complex hierarchical cascade, which includes the *gacA/gacS* (a two component signal transduction system) regulon, and two QS circuits encoded by the *phzR/phzI* and the *csaR/csaI* regulons. *PhzR/phzI* regulates the production of the antibiotics phenazine-1-carboxylic acid, and 2-hydroxy-phenazine (Fig. 2), whereas *csaR/csaI* regulates currently unknown aspects of the cell surface. Mutations in either *gacS* or *gacA* dramatically reduce production of many antimicrobial metabolites as well as production of acyl-homoserine-lactone signal molecules (synthesized by *phzI* and *csaI* gene products), implying that the *gacA/gacS* regulon is above the *phzR/phzI* and the *csaR/csaI* regulon in the hierarchical cascade. Mutations which result in QS defects in *P. aureofaciens* therefore include those in *gacA/gacS*, *phzR/phzI,* and *csaR/csaI*.

Aims

In the present exercise you will use miniTn*5* transposon mutagenesis to generate a *P. aureofaciens* mutant library, and you will screen for mutants with altered AHL profile through extracellular protease activity assay. The AHL profiles of the *P. aureofaciens* wild type and potential QS mutants will be characterized by the use of microtiter based bioassays. Another aim of the exercise is to isolate novel QS inhibitors by the use of special bioassays, and to study the effect of these QS inhibitors on *P. aeruginosa* virulence factor expression

Overview of experimental procedures in Exercise 4



Strains for Exercise 4

For part I:

Donor: MH379 (*E. coli* S17-λpir / pUTminiTn5-Gm), Ap^R, Gm^R (Incubate at 37ºC)

Helper: SM1279 (*E. coli* HB101/pRK600), Cm^R (Incubate at 37ºC)

Recipient: JB979 (GFP tagged *P. aureofaciens* ATCC13985, *wt*), Km^R, Cm^R (Incubate at 30ºC, 48 h)

AHL sensors: CV026 (*Chromobactrium violaceum, I*-mutant), Km^R (Incubate at 30ºC, 48 h)

MH281 (*Agrobacterium tumefaciens*, pDSK519-*traR*, *traG::lacZ*), Gm^R (Incubate at 30ºC)

JB525 (MT102 / pJBA132 (*luxR*-P_{luxR} –P_{luxI} –*gfp*(ASV)), Tc^R (Incubate at 30ºC, 48h)

Ref. strains: JB977 (GFP tagged *afmI(csaI)* mutant of *P. aureofaciens* ATCC13985), Km^R, Cam^R (Incubate at 30ºC, 48h)

JB975 (GFP tagged *gacS* mutant of *P. aureofaciens* ATCC13985), Km^R, Cam^R (Incubate at 30ºC, 48h)

For part II:

CVO26: *Chromobacterium violaceum*, AHL^-, $Km^{R\text{-}50\gamma}$ (Incubate at 30ºC, 48h)
MH155: *E. coli* /pUCP22NotI-P_{lasB}*::gfp*(ASV) P_{lac}*::*lasR, $Gm^{R\text{-}20\gamma}$ (Incubate at 37ºC)
JB357: *E. coli* /pUC18NotI-P_{luxI}*::gfp*(ASV) P_{luxR}*::luxR*, $Ap^{R\text{-}100\gamma}$ (Incubate at 37ºC)

PART I. Construction of QS-deficient mutants

Day 2 (Tuesday)

Transposon mutagenesis - Triparental matings.

Materials

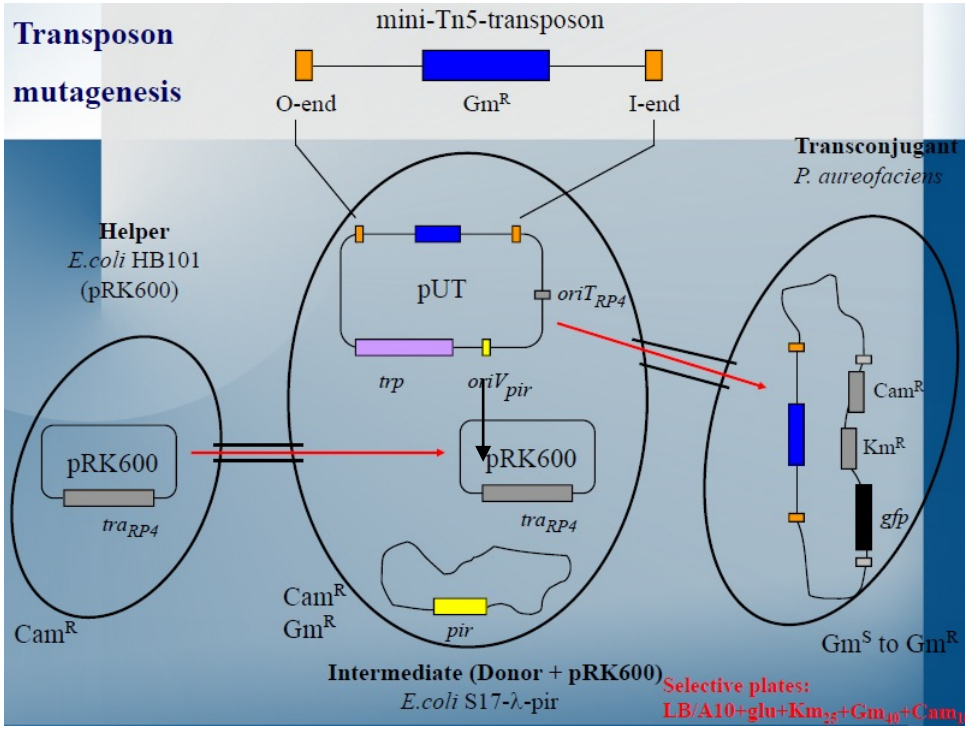
Over night culture of JB979
Over night culture of MH379
Over night culture of SM1279
3x 2 mL Eppendorph tubes
3x 10 mL 0.9 % NaCl for wash
2x LB plates **(Dry plates at 37°C for 30-40 min before use – This procedure is used every time)**

1. Harvest 2 mL over night culture of JB979 in a 2-mL Eppendorf tube by centrifuging for 2 minutes at 7,000g. Discard supernatant.
2. Harvest another 2 mL JB979 in the same tube. Centrifuge like before, and discard supernatant. Thus you end up with cell mass from 4 mL cell culture in one tube.
3. Harvest 2 mL over night culture of MH379 in a 2-mL Eppendorf tube. Centrifuge (2 min, 7,000g) and discard supernatant.
4. Harvest another 2 mL MH379 in the same tube. Centrifuge like before, and discard supernatant. Thus you end up with cell mass from 4 mL cell culture in one tube.
5. Harvest 2 mL over night culture of SM1279 in a 2-mL Eppendorf tube. Centrifuge (2 min, 7,000g) and discard supernatant.
6. Harvest another 2 mL SM1279 in the same tube. Centrifuge like before, and discard supernatant. Thus you end up with cell mass from 4 mL cell culture in one tube.
7. Wash the pellets twice with 1 mL 0.9 % NaCl (add 1 mL 0.9 % NaCl, vortex, centrifuge and discard supernatant). This is for removing the antibiotics.
8. Resuspend in 1 mL 0.9 % NaCl. Vortex.
9. For the conjugation, mix 0.5 mL MH379, 0.5 mL SM1279 and 1 mL JB979 in a new 2-mL Eppendorf tube. Spin down the cells by centrifugation (2 min, 7,000g).
10. Resuspend the pellet in 400 µL LB and spot 4 x 100 µL of the suspension onto 2 dry LB-plates (2 conjugation spots/plate). **Do not spread the spots! Do not turn the plate upside-down!**

11. Incubate the plates over night at **37°C (IMPORTANT)**.

The background of transposon mutagenesis (Fig. 3).

Mutants are produced in the following way. *E. coli* carrying a plasmid that contains a tranposon, a transposase, a lambda-pir-dependent origin of replication and an antibiotic selection marker are mated with wild type *P. aureofaciens* JB979. Because JB979 is lambda pir-, the transferred transposon plasmid is not replicated in JB979. JB979 transposants are selected on media containing antibiotics that select for the presence of the transposon and that select against *E. coli*.



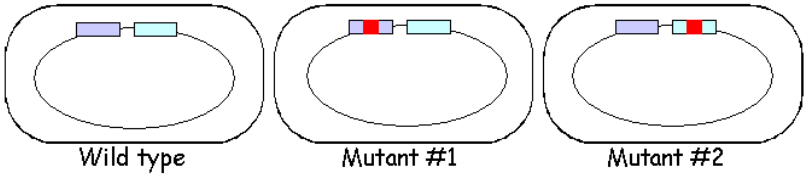


Figure 3. Transposon mutagenesis.

The background of Identification of Mutated Gene (Fig. 4).

The genomic DNA sequence adjacent to the transposon in each insertion mutant identifies the gene disrupted by the transposon. To determine the sequence of the adjacent DNA, two rounds of PCR are conducted. In the first round, a 5' primer specific to the transposon and a 3' ARB1 primer which contains a stretch of random nucleotides and a stretch of invariable nucleotides, are used to amplify genomic sequences. To enrich for genomic sequences adjacent to the transposon, a second round of PCR is performed using a nested 5' primer specific to the transposon sequence and a 3' ARB2 primer that anneals specifically with the invariant sequence present in the ARB1 primer. After cleaning up the ARB2 PCR reaction, another transposon-specific primer is used to sequence the Arbitrary PCR products. Subsequent bioinformatic analysis of the resulting sequences identifies the genomic locus adjacent to the transposon insertion.

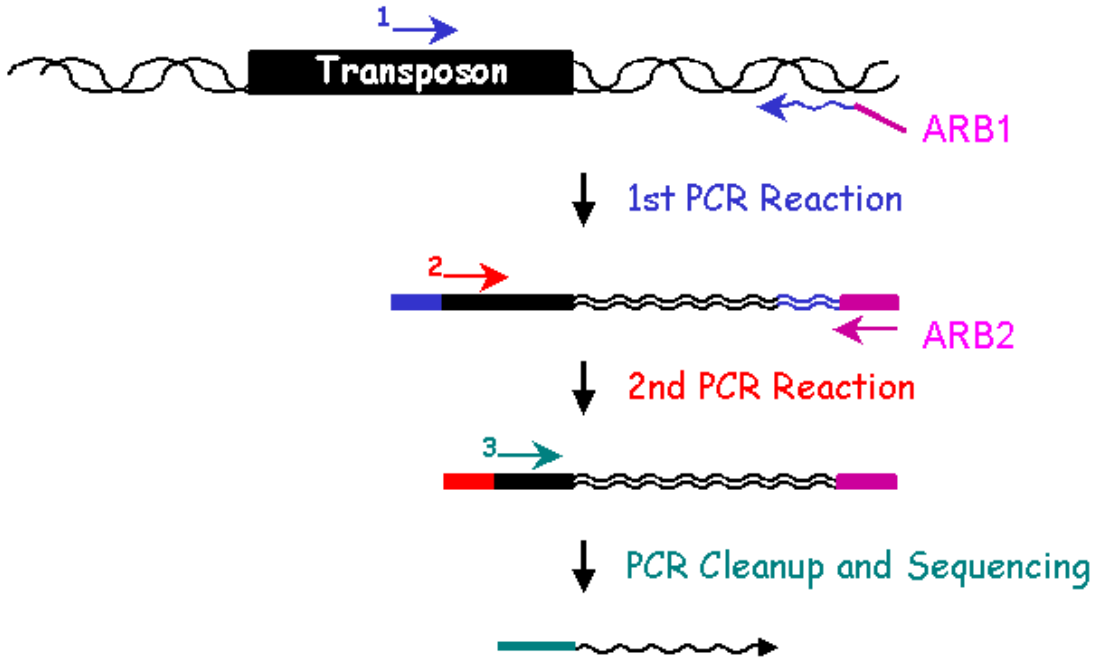


Figure 4. Arbitrary PCR Methodology: PCR Reaction 1 with primers 1 and ARB1 amplifies genomic DNA adjacent to the transposon. PCR Reaction 2 with nested primer 2 and ARB2 specifically enriches for the desired products from PCR Reaction 1. Products from PCR Reaction 2 are sequenced directly using primer 3.

Day 3 (Wednesday morning)

Plating of transconjugants on selective plates.

Materials

1x 2 mL Eppendorf tube

2x 10 mL 0.9% NaCl

6x LB Km25-Gm40-Cam10 plates

1. Gently scrape off the 4 conjugation spots with a streak (avoid collecting agar).
2. Add 1 mL of 0.9 % NaCl and resuspend the conjugation spots by pipetting gently.
3. Transfer the liquid from the 2 plates to a new 2-ml Eppendorf tube.
4. Spin the cells down (2 min., 10,000 g) and discard the supernatant.
5. Wash the cells **twice** with 1 mL 0.9 % NaCl (add 1 mL 0.9 % NaCl, vortex, centrifuge and discard supernatant).
6. Resuspend the pellet (conjugation mix) in 1 mL of 0.9 % NaCl. Vortex.
7. Spread 100 µL of the washed conjugation mix onto 6 LB Km25-Gm40-Cam10-plates (selective plates) separately.
8. Incubate plates at 28°C
9. The remaining 400 µL conjugation-mix is **stored** in the freezer for later use.

Please remember to bring samples next Monday for the part II of the excise quorum sensing inhibition! This sample can consist of leaves from your pot plant, fresh vegetables, herbs or herb medicines or whatever you can think of (especially some thing with flavor such as white garlic, ginger and so on).

Day 8 (Monday)

Phenotypic screening of transconjugants.

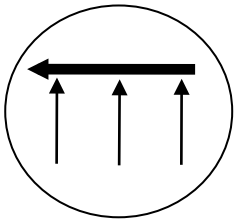
Materials

- Sterile tooth picks
- Plates with JB979, JB977, JB975, CVO26, and JB525
- ABTG + Cas-Amino Acids (0.5% glucose and 0.5% Cas-AA) plates
- Skim Milk Plates

In order to determine the AHL phenotypes of all the isolated mutants we will initially perform cross feeding experiments on agar plates between the isolated mutants and two different AHL monitor strains called CV026 (produces a purple pigment (violacein) in the presence of C4, C6, and to some extent C8 AHLs) and JB525 (produces GFP (green fluorescent protein)) in the presence of C6, C8, C10 and C12 AHLs).

1. Find and mark 20 white colonies (phenazine-deficient mutants) from the transconjugant plates with a marker pen.
2. Using a streak or a toothpick, cross-streak the selected mutants against CV026 (AHL monitor strain), by forming T's on LB plates. Streak monitor strain across the upper

part of the plate. Streak up the mutants from the opposite part towards the sensor strain **WITHOUT** touching the sensor strain, but **VERY CLOSE TO** (see picture). **Remember to change toothpick after every streak.**



3. Using a toothpick, cross streak the mutants of selected transconjugants against JB525 (another AHL monitor strain), by forming T's on ABTG-CasAA-plates (see picture). **Remember to change toothpick after every streak.**
4. Likewise, cross-streak JB979, JB977, and JB975 against CV026 by forming T's on LB plates and against JB525 by forming T's on ABTG-CasAA-plates as controls.
5. Use sterile toothpicks and randomly pick up to 60 white colonies and streak to 3 Skim Milk Plates (20 streaks on each plate, remember to keep some distance among the different streaks, make around 0.5 - 1 cm for each streak).
6. Use sterile tooth picks to pick JB979, JB977, and JB975 and streak to 1 Skim Milk Plates.
7. Incubate plates over night at 28ºC.

Day 10 (Wednesday)
Synthetic AHL feeding assay for Phenazine pigment production

Since QS molecule AHL are small diffusible molecules, we are going to check whether synthetic AHLs and AHL produced from neighbor cells could restore the phenotypes of selected QS mutants to the wild-type level.

1. Inspect the cross-feeding experiments, and note whether the potential AHL negative mutants give rise to an AHL negative phenotype (compare the impact of your mutants, JB979, and JB977 on the AHL sensor strains). Cross-feeding experiments involving CV026 are visually inspected for production of purple pigment. Cross-feeding experiments involving JB525 are inspected for production of GFP using a fluorescent microscope. All observations are noted in the result sheet.

2. Inspect the Skim Milk Plates and mark proteinase deficient mutants (which can not make a clearing zone as the JB975 mutant).
3. Inoculate 5 selected phenazine deficient QS mutants and two control strains (JB977 and JB975) in 14 ABTG-CasA tubes (inoculate two tubes (**a** and **b**) for each strain).
4. Add 1 µM HHL (final concentration) into tube **a** of each strain.
5. Add 1 µM BHL (final concentration) into tube **b** of each strain.
6. Incubate the tubes at 28ºC for 48 h with shaking.

Day 12 (Friday)
Observation of the AHL feeding assay

Check the phenazine pigment production of the AHL feeding assay and remember to take pictures for the report.

PART II. QUORUM SENSING INHIBITION

Day 8 (Monday)

Natural source library screening for QSI compounds

Materials

- CVO26: *Chromobacterium violaceum*, AHL$^-$, Km $^{R\text{-}50\gamma}$
- In this exercise you should use your own sample.

Sample preparation (morning)

All work with ethyl acetate is done in the fumehood!

1. Crush or chop your samples. You should have between 5-8 samples each group.
2. Transfer 5 g sample to a 100 ml flask.
3. Add 10 ml ethyl acetate. Mix well.
4. Leave the sample in the **fumehood** for a couple of hours. Shake the flask from time to time.
5. Filter the sample and transfer to a 100-ml beaker.
6. Evaporate most (but not all) ethyl acetate.
7. Make QSI indicator plates late in the afternoon, one of each type for each sample as described below.

CVO26 *Chromobacterium violaceum* is a Gram-negative bacterium commonly found in soil and water, which produces the characteristic purple pigment violacein. CVO26 is a pigment deficient mini-Tn5 mutant of *C. violaceum* in which pigment production can be restored by addition of *N*-hexanoyl-L-homoserine lactone (HHL). However, if a compound with quorum sensing inhibitory properties is added along with HHL, the production of violacein is abolished. Hence this biosensor can be used when screening for quorum sensing inhibitors, since a colourless zone around the well where the test sample has been applied, indicates the presence of an inhibitor.

CV026 indicator plates (afternoon)

1. Mix in the following order:
   200 µl o.n. culture of CV026,
   50 µl kanamycin,
   10 µl 10 mM HHL,
   50 ml melted (in the 45ºC water bath) LB-agar

2. Quickly after mixing (if not the agar will solidify), prepare two indicator plates. Wait 30 minutes.
3. After the two sets of plates have solidified, punch a hole for each of your samples (max 5 samples pr. plate) in the agar of each plate using the butt end of a 1000 µl pipette tip.
4. Add 100 µl of each of your extracts in one well.
5. Add 100 µl of penicillic acid (10 mM) in one well.

6. Add 100 µl of 4-nitropyridine-N-oxide (10 mM) in another well.
7. Plates are incubated 30ºC (plates are NOT incubated upside-down!).
8. Store the remaining extracts, HHL and kanamycin at -20ºC. Remember to write your names/team number on them.

Day 9 (Tuesday morning)

Dose-response investigation and optimization of AHL concentration

Strains

- MH155: *E. coli* /pUCP22NotI-P_{lasB}::*gfp*(ASV) P_{lac}::lasR, $Gm^{R\text{-}20\gamma}$.
- JB357: *E. coli* /pUC18NotI-P_{luxI}::*gfp*(ASV) P_{luxR}::*luxR*, $Ap^{R\text{-}100\gamma}$.

The AHL monitor systems mentioned above are bacterial strains genetically engineered to give a "visible" response if signal molecules are present in the growth medium. Before the AHL monitor systems are employed their limits and specificity have to be determined. In this exercise detection limit and saturation point are found for two AHL monitor strains (Table 1) and four different AHLs (Table 2). The QS systems involved are the *las* system from *P. aeruginosa* and the *lux* system from *Vibrio fischeri*. Each quorum sensing system is represented as an AHL monitor. The monitors have the promoter of a target gene for the given system fused to the gene encoding unstable green fluorescent protein; Gfp(ASV). When the gene encoding Gfp(ASV) is fused to a promoter positively regulated by quorum sensing, the induction of the promoter increases the expression of Gfp(ASV). Hence, the elevated expression of the quorum sensing controlled gene can be measured as increased fluorescence. The addition of a quorum sensing inhibitor to this type of construct results in lowered expression of Gfp(ASV) to an extent proportional to its efficiency as quorum sensing inhibitor. A toxic compound would also lower the fluorescence, but by measuring the growth rate along with the fluorescence this can be accounted for. The two fusions are located on plasmids harboured by *E. coli* (as outlined in Fig. 6).

Table 1. The two AHL monitors employed.

Monitor	QS system	Target gene	Reporter gene
MH155	*las*	*lasB*	*gfp(ASV)*
JB357	*lux*	*luxI*	*gfp(ASV)*

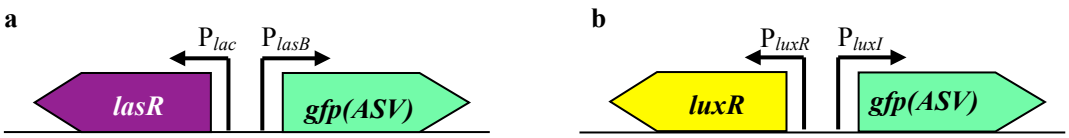
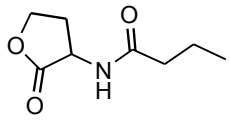
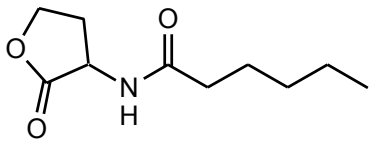
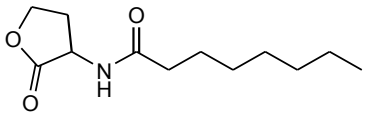
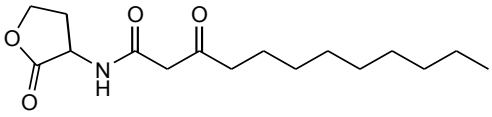


Figure 6. Schematic of the essential parts of **a** MH155. **b** and JB357.

Table 2. The four AHLs employed.

AHL	Abbreviation	Structure
N-Butanoyl-L-HSL	BHL	
N-hexanoyl-DL-HSL	HHL	
N-octanoyl-DL-HSL	OHL	
N-(3-oxo)-dodecanoyl -L-HSL	OdDHL	

Assay for determination of dose-response curves for the two monitor strains and different AHLs

We are going to use 1:2 serial dilutions in this assay.

"Serial dilution" - This term is frequently used and refers to a "multiple" dilution problem. In other words, an initial dilution is made and then this dilution is used to make a second dilution, and so on. For example, a 1:2 serial dilution is made using a 150 µl volume of sample. This expression indicates that 150 µl of sample is added to 150 µl of H_20 and then mixed. This initial dilution is 1:2. Then, 150 µl of this dilution is added to 150 µl of H_20 further diluting the sample. This same process is continued (Fig. 7).

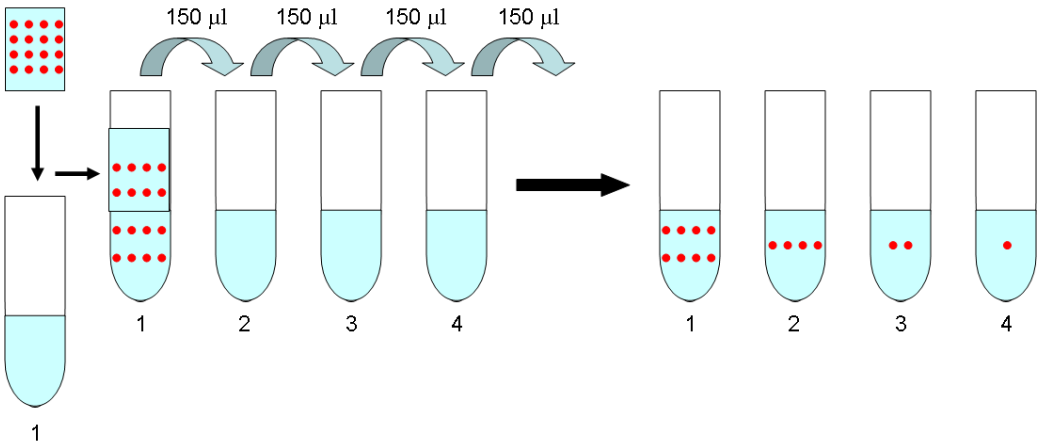


Figure 7. Illustration of 1:2 serial dilusion method.

- Mix 90 ml BT, 10 ml A10, 2.5 ml 20% glucose and 2.5 ml 20 % Cas-amino acids.
- Add 150 µl media to all 96 wells of a ("black") microtiter plate using a multipipette.
- Add 130 µl additional media to the wells in the first column (column 1)
- Add 20 µl of signal molecule to the wells in the first column; BHL in row A+E, HHL in row B+F, OHL in row C+G and OdDHL in row D+H.
- Make **1:2 serial dilutions** in columns 1-11 by transferring 150 µl from column1 to column 2 and so on. **The excess 150 µl from column 11 is discarded**. Column 12 is left as reference, that is, AHLs are not added to the wells in this column.
- Mix 4 ml ON monitor strain and 11 ml medium in a 15 ml tube (do this for both monitor strains).
- Add 150 µl monitor strain from the 15 ml tube to each well. JB357 is added to all wells in row A-D, and MH155 is added to the wells in row E-H
- Put on a lid and incubate 3-4 hours at 37 ºC.
- Measure OD and fluorescence using the multi label plate reader.

Again, store the remaining signal molecules (BHL, HHL, OHL and OdDHL) at -20ºC. Store remaining glucose and cas-amino acids in the fridge (4°C). Make sure that your team number is written on the tubes.

Do the data analysis as soon as possible. You need the results for day 11's experimental work!!!

Data analyses

Draw dose-response curves for the two monitor strains showing relative fluorescence (fluorescence pr. OD) as a function of the concentration of the different signal molecules. Also find the relation between the fluorescence and the concentration of the different signal molecules as well as the relationship between OD and the concentration of the different signal molecules.

Characterize the two monitors:

- Are they broad or narrow spectrum?

- Which are their cognate AHLs? That is, which AHL activates the monitor strains?

- Which concentration of AHL is necessary to activate the monitor strains?

Day 10 (Wednesday)

Dose-response curves with quorum sensing inhibitors

Strains

- MH155: *E. coli* /pUCP22NotI-P$_{lasB}$*::gfp*(ASV) P$_{lac}$*::*lasR, Gm$^{R\text{-}20\gamma}$.
- JB357: *E. coli* /pUC18NotI-P$_{luxI}$*::gfp*(ASV) P$_{luxR}$*::luxR*, Ap$^{R\text{-}100\gamma}$.
- ON culture *Pseudomonas aeruginosa* PA14.

This part is focused on creating a dose-response curve in the presence of quorum sensing inhibitors. You will use the optimal type of AHL determined by the dose-response optimization. Use patulin, 4-nitropyridine-N-oxide, and penicillic acid as QSI compounds. Also test the extract(s) you made for quorum sensing inhibitory properties.

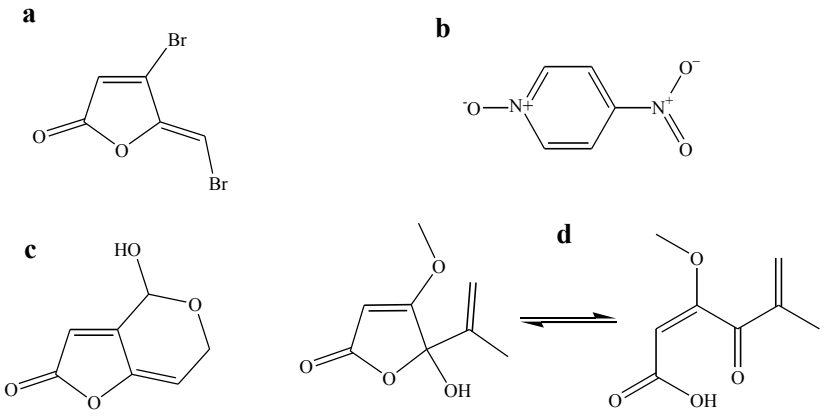


Figure 8. Quorum sensing inhibitory compounds **a.** the halogenated furanone C30, **b.** 4-nitropyridine-*N*-oxide **c**. patulin **d.** penicillic acid.

Assay for determination of dose-response curves in the presence of QSIs

- Mix 90 ml BT, 10 ml A10, 2.5 ml 20% glucose and 2.5 ml 20 % Cas-amino acids.
- Add 150 µl media to all 96 wells of a ("black") microtiter plate using a multi-pipette.
- Add 130 µl additional media to the wells in the first column (column 1)
- Add 20 µl of QSI compound to the wells in the first column; patulin in row A+E, 4-nitropyridine-N-oxide in row B+F, penicillic acid in row C+G and your own extract in row D+H.
- Make **1:2 serial dilutions** in columns 1-11 by transferring 150 µl from column1 to column 2 and so on. **The excess 150 µl from column 11 is discarded.** Column 12 is left as reference, that is, QSIs are not added to the wells in this column.

- Mix 2 ml ON monitor strain and 6 ml medium in a 15 ml tube and store for later use (do this for both monitor strains).
- Add 150 µl of the AHL you found in the Dose response curve experiment from yesterday to the 15-ml tube with monitor strain.
- Add 150 µl of the monitor strain-AHL mix to each well. JB357 is added to all wells in row A-D, and MH155 is added to all the wells in row E-H
- Put on a lid and incubate 3-4 hours at 37 ºC
- Measure OD and fluorescence in the multi-label plate reader.

Data analyses

Draw dose-response curves for the two monitors showing fluorescence (and OD) as function of concentration of the quorum sensing inhibitors. Find the highest concentration of each inhibitor, which does not have an impact on growth (i.e. OD is not affected compared to the reference). **Please write down these concentrations in your reports.**

Inoculation for virulence factor assays (Important!)

1. Inoculate 3 10-ml LB tubes using 5 µl of a *Pseudomonas aeruginosa* PA14 ON culture.
2. To one tube 20 µl patulin is added and to another 20 µl penicillic acid is added. The third serves as an untreated control. Remember to write team number and content on all tubes.

Day 11: (Thursday) Quorum-sensing regulated virulence factor inhibition assays.

Strains

- Plate with *Pseudomonas aeruginosa* PA14.

Rhamnolipid emulsification activity assay.

> Rhamnolipid is a biosurfactant, do you have any ideas how this compound will harm host issues during the infections? On the other hand, do you have any ideas how this compound can be applied in the industry and pollution controlling?

1. Shake the ON-culture tubes gently for 10 seconds and then transfer 1.5 ml of ON culture of the three inoculated tubes from Day 10 to three new centrifuge tubes separately.
2. Centrifuge at 10,000 rpm for 2 min.
3. Transfer 0.5 ml supernatant from the three centrifuge tubes to three 15-ml yellow-cap tubes.
4. Add 1 ml n-hexadecane to these three tubes separately.
5. Screw the yellow caps and vortex the 10 ml yellow cap tubes for 2 min.
6. Allow these yellow-cap tubes to stand on the table for 1 hour before inspection.

Emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as a percentage.

Swarming is a rapid mode of surface translocation. The bacterium *Pseudomonas aeruginosa* PA14 is able to swarm on top of 0.6% agar plates. This process is dependent not only on synthesis and operation of flagella, but also on production of a surface active compound – the surfactant Rhamnolipid. The gene encoding serrawettin synthase, *RhlA*, is controlled by the *rhl* quorum sensing system. If the quorum sensing system is inhibited, PA14 is unable to swarm. This can be employed as a screening for quorum sensing inhibitors. Here, two quorum sensing inhibitors, the halogenated furanone patulin and penicillic acid (Fig. 7) are tested for inhibition of QS controlled surface motility.

Swarmming assay

1. Mix 10 ml A10, 1 ml 20% glucose and 1 ml 20% Cas-amino acids.
2. Melt 90 ml BT agar (which contains only 0.6% agar) by microwave oven.
3. Mix the melted agar with the mix from step 1. Now you have swarm medium (ABTG-casa with 0.6% agar).
4. Add 10 ml melted swarm medium to 6 of the 15-ml yellow-cap tubes.
5. Quickly afterwards, add the penicillic acid into their 6 yellow cap tubes as indicated in the table below (stock solution is 10 mM).

Tube number	**1**	**2**	**3**	**4**	**5**	**6**
Test compound (µl)	100	50	10	5	2.5	0
Final concentration (µM)	100	50	10	5	2.5	0

6. Gently shake the tubes and poor the medium from the 6 tubes into 6 petri dish plates separately.
7. After the agar has solidified, the plates are left to dry 20 minutes without lid just on the table (**not** in the incubator).
8. Pick one colony of PA14 with a toothpick and inoculate it on the surface (not inside the agar) in the center of each plate.
9. Put on lids and incubate over night at 30ºC (**not** upside down!).

Day 12 (Friday)

Observe swarming assay results

Take pictures of the results and write your reports.